

## Basic Investigation

### Gualou Xiebai Banxia Decoction (瓜蒌薤白半夏汤) Inhibits NF-kappa B-dependent Inflammation in Myocardial Ischemia-reperfusion Injury in Rats

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**Objective:** To evaluate the myocardial protective effect of Gualou Xiebai Banxia decoction (瓜蒌薤白半夏汤 GXBD) and explore the mechanisms of inhibition of NF-kappa B activation and blockade of inflammatory responses induced by ischemia-reperfusion in rats.

**Methods:** Twenty-four Sprague Dawley (SD) rats were randomly divided into three groups. Rats in the treatment group received GXBD (13 g crude drug/kg) for three weeks, while rats in the model control and normal control groups received equal volumes of distilled water. On the 22nd day, rats in the ischemia-reperfusion (I/R) control and GXBD-treated groups underwent 30 min occlusion of the left anterior descending (LAD) coronary artery, followed by 120 min reperfusion. Electrocardiogram was recorded, and the activities of cardiac enzymes, cytokines, and NF-κB were assessed after I/R.

**Results:** Compared with the I/R control group, GXBD treatment restored the activity of the specific myocardial-injury marker creatine kinase (CK) and lactate dehydrogenase (LDH), and inhibited the inflammatory response involving the nuclear factor-κB (NF-κB) pathway, including down-regulation of interleukin (IL)-1β and IL-6, and up-regulation of IL-10 gene expression.

**Conclusion:** GXBD strongly reduced myocardial impairment in our I/R model, including inhibition of NF-κB activation and inflammatory cytokine responses.

**Keywords:** *Gualou Xiebai Banxia decoction (GXBD); ischemia-reperfusion; myocardial infarction; inflammation*

Myocardial ischemia-reperfusion injury (MI/RI) is a common clinical entity. To limit the degree of myocardial injury, it is essential to ensure rapid recovery of blood flow in ischemic tissues and to quickly relieve ischemia-reperfusion injury. Previous studies have shown that MI/RI is a complicated process involving multiple pathways.<sup>1-2</sup> Further, despite the numerous mechanisms proposed to underlie the pathology in MI/RI, including free radical injury, calcium overload, and injury of vascular endothelial cells, these processes do not account for all pathological changes observed. Interestingly, there is increasing evidence from animal experiments and clinical studies of an important role for inflammatory responses in MI/RI.<sup>3-4</sup>

Discovery of the protective effect of Chinese Medicine on MI/RI was an important advance for integrated traditional and western medicine. Thus, it is critical to systematically screen effective herbs to investigate their treatment mechanisms for MI/RI in order to facilitate the prevention and cure of this disease, as well as provide a further understanding of the practical clinical value and underlying mechanisms of traditional Chinese herbal prescriptions. Gualou Xiebai Banxia decoction (GXBD) comes from *Jin Gui Yao Lue* (金匱要略 Medical Treasures of the Golden Chamber) written by Zhang Zhong-jing, a famous medical practitioner of the Eastern Han Dynasty. GXBD has been reported to promote

“yang energy” and remove obstruction, eliminate phlegm, and soothe chest oppression, and is widely used to treat chest stuffiness and other cardiovascular diseases in the clinic. There is also increasing evidence that GXBD may be efficacious for coronary heart disease, with experimental studies demonstrating that GXBD can significantly ameliorate atherosclerosis and improve ischemia-reperfusion injury, resulting in improved myocardial function. However, the underlying mechanisms remain unclear.<sup>5</sup>

In the present study, we examined the role of GXBD in modulating inflammatory responses, in particular the NIK/NF-κB signal transduction pathway, in an experimental ischemia-reperfusion model of myocardial infarction (MI) in rats.

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## MATERIALS AND METHODS

### Animals

In this study, 24 male SD rats weighing  $300 \pm 20$  g were used. Animals were provided by the Experimental Animal Center of the Chinese Academy of Military Medical Sciences (Certificate No. SCXK-[Military] 2007-004). Animals were housed under constant conditions of  $23 \pm 1$  °C,  $40 \pm 5\%$  humidity, with a 12:12 h light-dark cycle, and had free accesses to feed pellets and tap water. All animals were cared for in accordance with the policies and guidelines by Ethic Committee for Animal Use in the Shanghai Institute of Medical Materials.

### Preparation of GXBD

Gua Lou (Fructus Trichosanthis), Xie Bai (Allium macrostemon Bunge), and Ban Xia (Rhizoma Pinelliae) were purchased from Bozhou Fangyitang Traditional Chinese Medicine Co., Ltd. (Anhui, China) in October 2008, and were identified as *Trichosanthes kirilowii* Maxim., *Allium macrostemon* Bge., and *Pinellia tuberifera* Tenore., respectively, by engineer Xi-Rong He (Institute of Traditional Chinese Medicine, China Academy of Chinese Medical Sciences). The decoction of GXBD was composed of Gua Lou (24 g), Xie Bai (9 g), and Ban Xia (12 g). The GXBD was concentrated to 1.3 g drug per milliliter. According to the clinical dosage for a 70 kg adult, we converted the dosage for rats to 1.3 g crude drug per 100 g body weight.

### Induction of Myocardial Ischemia-reperfusion Injury

Before the operation, the rats were fasted for 24 h with free access to water. After anesthesia with intraperitoneal injection of 20% urethane (0.5 mL/100 g) in the supine position, the limbs were fixed in the supine position, the hairs on neck and chest were shaven, and the surface ECG (lead II) was recorded. Oxygen was given via the trachea with a respiration machine (respiration rate 70/min, respiration-to-expiration ratio 2:1, tidal volume 9 mL/kg). The muscle tissue was bluntly dissected, and the thoracic cavity was opened and carefully dissected through the pericardial sack. The left anterior descending branch of the coronary artery, which was between the left atrial appendage and the pulmonary conus, was identified and ligated with a 50 monofilament nylon (a small polyethylene pipe was put between the myocardia and the monofilament nylon).

ECG was recorded when cyanosis and protrusion appeared on the wall of the ischemic myocardium, and myocardial ischemia formation showed saddleback-type ST segment elevation and a significant T-wave increase. Thirty minutes later the ligation was removed to allow reperfusion of the ischemic coronary artery. The thoracic cavity was closed and reperfusion was performed for 120 min. During this time, rats that showed an ST recovery segment more than 50% on ECG were considered a successful model.

### Experimental Protocol

Twenty four SD rats were randomly divided into three groups: a sham-operation control group, an I/R control group, and a GXBD-treated group (eight rats per group). The rats were orally administered saline (1 mL/100 g body weight) or drug once per day for 3 weeks before they were subjected to I/R injury. I/R operation was performed at 2 h after the last administration. In the sham-operation control group, the rats were exposed to the same experimental conditions without ligation. Blood was taken from the abdominal aorta after 2 h reperfusion, and the hearts were quickly removed and rinsed with pre-cooled saline. A piece of the left coronary artery was dissected on an ice tray, weighed, and then rapidly frozen in liquid nitrogen and preserved at -80 °C.

### Pathomorphological Observation of the Myocardial Tissues

Myocardial tissues in the cardiac apex of rats were fixed in 3% glutaraldehyde at 4 °C for 4 h, post-fixed in 1% osmium tetroxide at 4 °C for 1.5 h, and then embedded with epoxy. After staining with 3% lead citrate and uranyl acetate, ultrathin sections were examined under by transmission electron microscopy.

### Determination of NIK, IKK $\beta$ , and I $\kappa$ B $\alpha$ Protein by Western Blotting

Myocardial specimens (100 mg) were cut into small pieces and mixed with lysate, and the suspension was homogenized and centrifuged. Protein concentration was determined by the Bradford method, and tissue was preserved at -80 °C prior to testing. A 50  $\mu$ g of tissue from each group was used for 15% SDS-PAGE, which was transferred to a PVDF membrane and then blocked in 5% de-fatted milk powder for 1 h at room temperature. The membranes were incubated in NIK, IKK $\beta$ , and I $\kappa$ B $\alpha$  antibodies (Cell Signaling Technology Boston, MA, USA) diluted at 1:2,000 in TBST buffer at 4 °C overnight. Membranes were washed twice in TBST (10 min each), followed by horseradish peroxidase (HRP)-labeled secondary antibody (Santa Cruz, CA, USA) at 4 °C for 1 h. Membranes were rinsed twice in TBST (10 min each), and then stained with electrochemiluminescence. Image analysis was performed with ImageMaster VDS software (Amersham Pharmacia Biotech, Uppsala, Sweden) using  $\beta$ -actin as an internal control, and the relative density calculated to determine the relative protein concentration.

### Quantitation of NF- $\kappa$ Bp65 mRNA by RT-PCR

Total RNA was extracted from the tissues using TRIzol (Sangon, Shanghai, China), and cDNA was synthesized according to the manufacturer's instructions. PCR was performed in GeneAmp 9600 (Perkin Elme, Waltham, MA, USA) with cDNA as template by adding TaqDNA, 10 $\times$ Buffer, MgCl<sub>2</sub>, dNTP, and forward and reverse primers for GAPDH and NF- $\kappa$ Bp65 (Sangon Shanghai, China). The primer sequences were: NF- $\kappa$ Bp65, forward primer 5'-ATGGACGATCTGTTCCCT-3', and reverse primer 5'-GGTGCCTAGTGGTATCT-3'; GAPDH,

forward primer 5'-CCTTCATTGACCTCAACTA CATG-3', and reverse primer 5'-CTTCTCCAT GGTGGTGAAGAC-3'. The PCR parameters were 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 45 s, then an extension of 72 °C for 7 min. The PCR products were 288 bp and 216 bp long, respectively. The PCR amplified products were determined by agarose gel electrophoresis, and the bands of the target gene were analyzed by ImageMaster VDS software (Amersham Pharmacia Biotech, Uppsala, Sweden). The integrated optical density (IOD) was recorded, and the relative IOD ratio of NF-κBp65 mRNA was expressed as target gene/GAPDH.

### Determination of IL-1β, IL-6, and IL-10 Activity

The serum concentrations of IL-1β, IL-6, and IL-10 were determined using the ELISA detection kit (R&D Systems, Minneapolis, MN, USA). The OD value was measured in an Enzyme-labeled Multiskan (Thermo Scientific, Waltham, MA, USA).

### Determination of CK-MB and LDH Levels

Blood samples were collected to determine plasma creatine kinase (CK) and lactic dehydrogenase (LDH) release. All samples were assayed using the Full Automatic Biochemical Analyzer (HITACHI, Tokyo, Japan). The reagents were provided from Beijing Zhongsheng Beikong Biotechnology Co., Ltd (BIOSINO, Beijing, China).

### Statistical Analysis

Data were expressed as mean±SD. Group means were compared by one-way ANOVA. If the requirements of variance homogeneity were met, the comparison among groups was performed with the LSD method. Alternatively, the Tamhane's T2 method was used for pair-wise comparisons. Compared with the sham operation group \* $P<0.05$ , \*\* $P<0.01$ ; compared with the model group # $P<0.05$ , ## $P<0.01$ .

## RESULTS

### Surface Electrocardiogram Observation

ECG was recorded for assessment of formation of myocardial ischemia and reperfusion. After ligation, ECG revealed a significant increase in saddleback-type ST segment and T-wave. Following reperfusion, ECG showed recovery of the ST segment. Thus, this characteristic ECG change indicated a successful operation. In the test groups, treatment with GXBD significantly reduced these effects (Figure 1).

### Morphological Changes in the Rat Myocardium

In the sham operation group the myocardial fibers were arranged regularly, the bands of sarcomere had a clear structure, rows of mitochondrial with a moderately dense matrix and dense mitochondrial cristae were observed in myofilaments, a small amount of glycogenosome was scattered between the mitochondria and the sarcomere, the sarcoplasmic reticulum structures were completely normal, the nucleus was oval-shaped at the center of the

cell, and there were abundant pinocytotic vesicles in endothelial cells of interstitial blood vessels. In the model group the myocardial fibers were arranged irregularly, there was cell matrix edema and myofibril cracking or fusion, the internal and external mitochondrial membrane disappeared, the mitochondrial crest exhibited a floccus change, the membrane of endothelial cells in interstitial blood vessels were damaged, vacuolar denaturation was evident, and the pinocytotic vesicles were decreased. In the GXBD group there was evidence of mitochondrial hyperplasia in myocardial cells, mild vacuolar denaturation with increased density of the matrix and myofibril cracks was apparent, and there were no significant interstitial changes (Figure 2).

### The Effects of GXBD on NIK, IKKβ, and IκBα Protein Expression in the Rat Myocardium

NIK protein expression was weak in the sham operation group, but was significantly up-regulated in the model group ( $P<0.01$ ; Figure 3). NIK protein expression showed a non-significant decrease in the GXBD group compared to the model group ( $P>0.05$ ). IKKβ protein expression in the model group was significantly higher than that in the sham operation group ( $P<0.01$ ; Figures 3 and 4). There was a significant decrease in IKKβ protein expression in the GXBD group compared to the model group ( $P<0.05$ ). In the model group, IκBα protein expression was significantly lower than that in the sham operation group ( $P<0.01$ ), while GXBD treatment significantly increased IκBα protein expression compared to the model group ( $P<0.01$ ).

### NF-κBp65 mRNA Expression in the Rat Myocardium

NF-κBp65 mRNA expression in the myocardium was very low in the sham operation group, but was significantly increased in the model group ( $P<0.01$ ). By contrast, the expression of NF-κBp65 mRNA in the GXBD group was significantly lower than that in the model group ( $P<0.05$ ).

### Effects of GXBD on Serum CK and LDH Levels

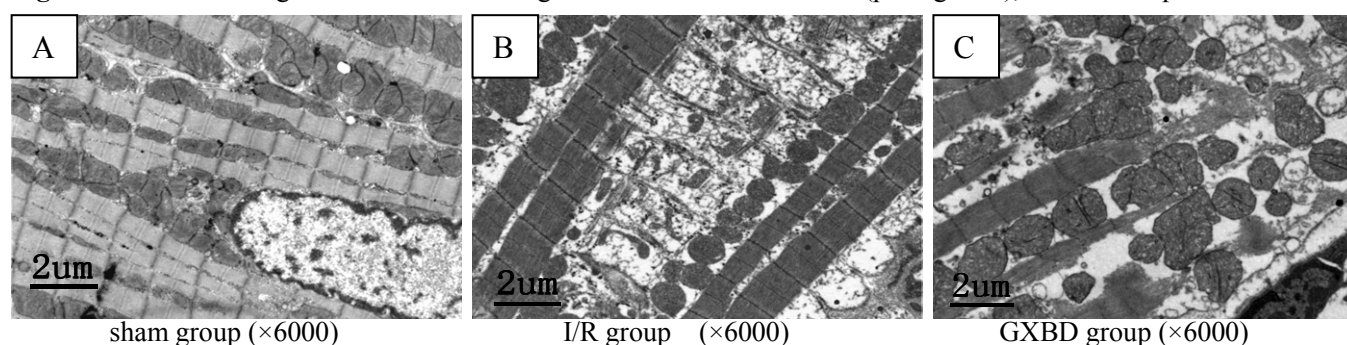
The serum concentrations of CK and LDH in the model group were significantly higher than those in the sham operation group after MI/RI ( $P<0.01$ ). There was a non-significant trend for decreased serum CK and LDH concentrations in the GXBD-treated group compared to the model group (Figure 5).

### Effects of GXBD on Serum IL-1β, IL-6, and IL-10 Levels

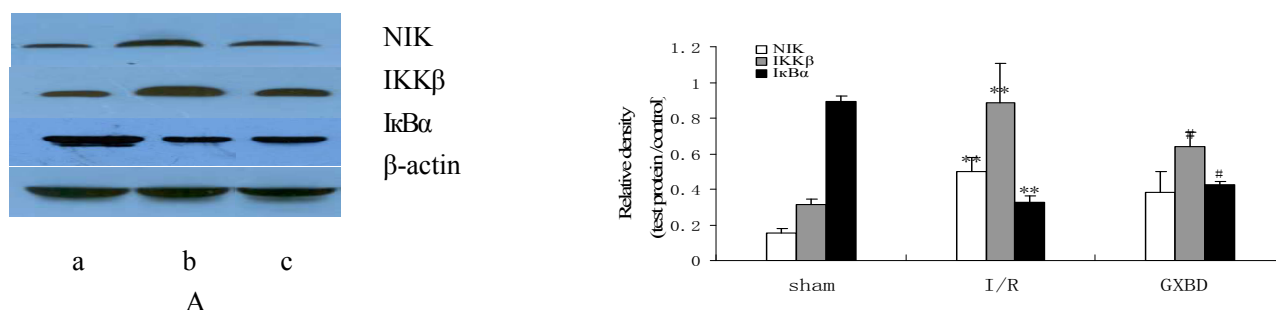
In the model group there was a significant increase in serum levels of IL-6 and IL-1β, and a significant decrease in serum levels of IL-10, compared to the sham operation group ( $P<0.01$ ). Pretreatment with GXBD significantly decreased IL-1 and IL-6 levels and increased IL-10 levels compared to the model group ( $P<0.05$ ; Figure 6).



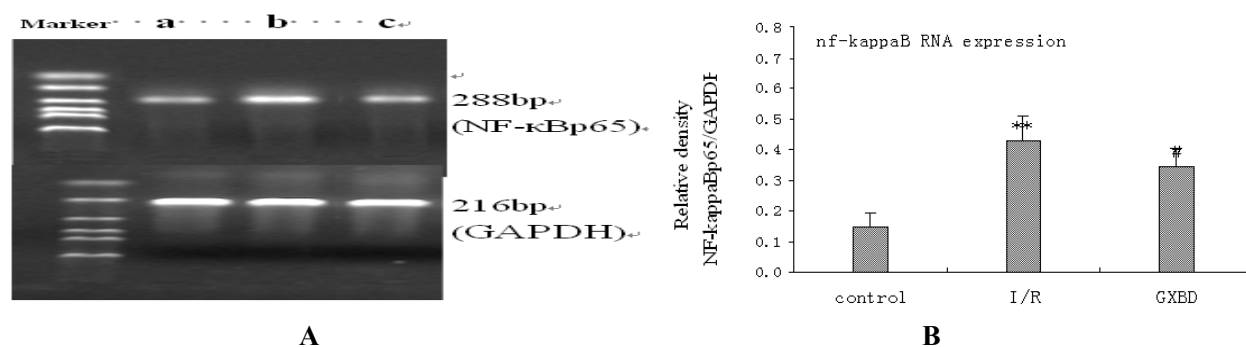
**Figure 1.** Electrocardiogram observation during the course from anesthesia (pre-ligation), ischemia to perfusion.



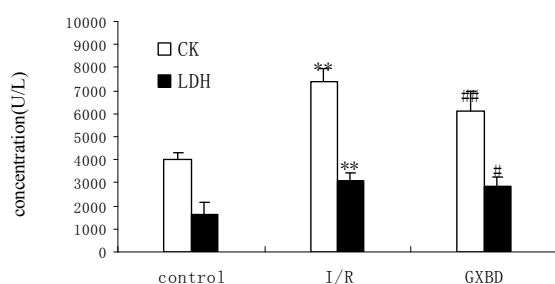
**Figure 2.** Ultrastructure changes of rat myocardia via electron microscope. A: sham control group; B: I/R model group; C: GXBD treated group.



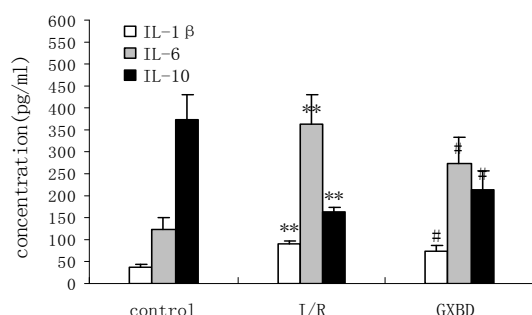
**Figure 3.** Western blotting test for IKKβ, IκBα and NIK protein expression in animals treated with GXBD decoction (A). lane a: sham operation group; lane b: I/R group; lane c: GXBD group. The density analysis of IKKβ, IκBα and NIK protein expression normalized with β-actin. (B). The data are expressed as mean ± SD ( $n=5$ ); compared with sham operation group \* $P<0.05$ , \*\* $P<0.01$ ; compared with I/R group # $P<0.05$ , ## $P<0.01$ .



**Figure 4.** The determination of NF-κBp65 mRNA in RT-PCR experiments. Lane a: sham operation group; Lane b: I/R group; and Lane c: GXBD group. The NF-κBp65 PCR and GAPDH products were 288 bp and 216 bp long, respectively (A). The relative density of NF-κBp65 mRNA was expressed as target gene/GAPDH (B). The data are expressed as mean ± SD ( $n=5$ ); compared with sham operation group \* $P<0.05$ , \*\* $P<0.01$ ; compared with model group # $P<0.05$ , ## $P<0.01$ .



**Figure 5.** The determination of CK, LDH in the plasma of different experimental groups. The results are expressed as mean  $\pm$  SD of eight different determinations in each experiment; compared with sham operation group \* $P$ <0.05, \*\* $P$ <0.01; compared with I/R group # $P$ <0.05, ## $P$ <0.01.



**Figure 6.** Determination of IL-1, IL-6 and IL-10 activity in the serum of rats in different experimental groups using ELISA test. The results are expressed as mean  $\pm$  SD of eight different determinations in each experiment. compared with sham operation group \* $P$ <0.05, \*\* $P$ <0.01; compared with I/R group # $P$ <0.05, ## $P$ <0.01.

## DISCUSSION

The present study demonstrated that GXBD treatment improved I/R-induced impairment in cardiac function and inhibited NF- $\kappa$ B activation. Furthermore, GXBD reversed the increase in cardiac enzymes (CK and LDH) and cytokines (IL-1, IL-10), and the decrease in IL-6, following I/R. The general markers of cardiac dysfunction in the model group including ST segment elevation in ECG and cardiac infarction were also alleviated with GXBD pretreatment.

The myocardial inflammatory response may be induced by myocardial ischemia, and reperfusion may aggravate the inflammation response. Our results showed that NF- $\kappa$ B activation was an important cause of MD/RI *in vivo*. The NF- $\kappa$ B shift was regulated by I $\kappa$ B subunit phosphorylation and degradation, which is the earliest point in the cascade of acute inflammation.<sup>6</sup> In the NF- $\kappa$ B signal transduction pathway, I $\kappa$ B is dissociated from the NF- $\kappa$ B complex, and NF- $\kappa$ B is translocated to the nucleus where it combines with specific sequences of KB. NF- $\kappa$ B is activated by inhibition of protein phosphorylation, and is regulated by IKK. It was previously demonstrated in

mammalian cells that IKK $\alpha$  and IKK $\beta$  can directly interact with I $\kappa$ B protein and participate in the process of phosphorylation of  $\kappa$ B $\alpha$ .<sup>7</sup> As a result, NF- $\kappa$ B is translocated into the nucleus to induce activation of factors involved in natural immunity and acquired immunity including pro-inflammatory factors (e.g., IL-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$ ), adhesion factors such as intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1). Adhesion and aggregation of neutrophils in the ischemic region can obstruct the vessel lumen under these inflammatory conditions.<sup>8</sup> The expression of adhesion molecules increases and attracts more neutrophils and lymphocytes, leading to injury to vascular endothelial cells and myocardial cells.<sup>9</sup> Therefore, NF- $\kappa$ B signaling is likely to regulate the pathological processes of MI/RI inflammation.

Our results showed that GXBD reduced injury to the myocardial ultrastructure, which plays an important role in MI/RI. Our experiments demonstrated that NIK and IKK $\beta$  protein expression in the model group were significantly upregulated compared with the sham operation group, while I $\kappa$ B $\alpha$  protein was significantly reduced. Expression of myocardial NF- $\kappa$ Bp65 mRNA was significantly increased in the model group. After myocardial ischemia-reperfusion, the serum levels of IL-6 and IL-1 $\beta$  in the model group were also significantly higher than those in the sham operation group, while the level of the anti-inflammatory cytokine IL-10 was significantly lower. These data partially confirm that up-regulation of NIK signaling molecules activates IKK $\beta$  and accelerates I $\kappa$ B dissociation to activate NF- $\kappa$ Bp65 transcription, which can regulate the expression of inflammatory molecules that promote the development of MI/RI. Our data also suggest that GXBD can inhibit this pathway, with decreased expression of NIK and IKK $\beta$  protein, and decreased phosphorylation and degradation of I $\kappa$ B $\alpha$  protein, resulting in inhibition of NF- $\kappa$ B activation. The decreased expression of IL-6 and IL-1 $\beta$  and increased expression of IL-10 observed with GXBD is likely a key mechanism of its protective action against MI/RI.

In conclusion, we demonstrated that GXBD can strongly reduce myocardial impairment, inhibit NF- $\kappa$ B activation, and modulate the balance between pro- and anti-inflammatory cytokine release following MI/RI. Future decomposed recipe studies are required to define the efficacy and effective components of this decoction.

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